

## REMARKS

This is in response to the non-final Office Action mailed July 10, 2008.

Currently pending are claim 38 and its dependent claims 21, 23-24, 35-37, 41, 50, 55-58, 61, 63 and 78-79. Claims 22, 25-27, 29, 42-49, 51-54, 59-60, 62 and 64-68 have been withdrawn as being based on non-elected species.

Claims 24, 38 and 41 are currently amended. Support for these amendments can be found throughout the specification, e.g., at paragraphs 34-35 of the application as filed. Claims 78-79 are added. Support for these claims can be found throughout the specification, e.g., at paragraph 35 of the application as filed. Claims 39-40 and 43-45 are cancelled without prejudice. In light of the foregoing amendments and the following remarks, Applicants submit that this application is in condition for allowance.

Applicants thank the Examiner for the courtesies extended during the telephonic interview on October 21, 2008, the Examiner's summary of which, as contained in the Interview Summary Form (PTOL-413), has been received.

### Claim Rejection – 35 U.S.C. § 112, First Paragraph

In the Office Action at page 3, claims 21, 23, 35-40, 50, 55-58 and 63 are rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the written description requirement. The Office Action alleges that “[t]he appearance of mere indistinct words (here the word ‘antagonist’ and ‘analog’) does not necessarily satisfy the written description requirement[]” (Office Action, p. 3). However, the Office Action states that “1- $\beta$ , 25-dihydroxyvitamin D and its corresponding homologs or isomers and calcium” do meet the written description requirement (Office Action, p. 4). Applicants respectfully traverse.

However, solely to expedite prosecution, Applicants have amended claim 38 to recite an “antagonist of calcitrophic hormone (1,25-(OH)<sub>2</sub>-D) activity selected from the group consisting of 1- $\beta$ , 25-dihydroxyvitamin D, a homolog of 1- $\beta$ , 25-dihydroxyvitamin D, an isomer of 1- $\beta$ , 25-dihydroxyvitamin D, and calcium[.]” This should obviate the Examiner’s rejection.

Claims 21, 23, 35-37, 50, 55-58 and 63 depend from claim 38 and are therefore allowable as depending from an allowable claim. In addition, new claims 78 and 79 recite particular sources of

calcium, and are also allowable as being dependent on an allowable claim. In view of the foregoing, Applicants respectfully request that this rejection be withdrawn.

**Claim Rejection – 35 U.S.C. § 102(a)**

In the Office Action at page 5, claims 21, 23, 38-39 and 57 are rejected under 35 U.S.C. § 102(a) as being unpatentable over an article by McCarthy in the March / April 2000 edition of Remedy (hereinafter "McCarthy").

In view of the attached inventor Declaration under 37 C.F.R. § 1.132, it is respectfully submitted that McCarthy is not prior art to the invention and that the publication disclosure was derived from the work of the inventors.

This application is a continuation-in-part of Application No. PCT/US01/27432, filed on September 4, 2001, which is a continuation-in-part of Application No. 09/654,357 ("the '357 application"), filed on September 1, 2000, now U.S. Patent 6,384,087 ("the '087" patent). The claim elements at issue are supported in the '357 application (see, e.g., page 6, lines 8-22 of the '357 application as filed). Accordingly, they are entitled to a priority date of September 1, 2000.

McCarthy was published in Remedy in the March/April 2000 edition, within one year before the priority date of the instant claims. McCarthy discusses research conducted under the direction of Dr. Michael Zemel at the University of Tennessee demonstrating that administration of foods containing calcium for one year resulted in an average weight loss of 11 pounds. In McCarthy, Dr. Zemel is quoted as saying that "[c]onsuming more [calcium] decreases production of a hormone called 1, 25-dihydroxyvitamin D, which stimulates fat production." The only part of McCarthy that discusses the effect of 1,25-dihydroxyvitamin D antagonists on fat or body weight is the section that discusses Dr. Zemel's work. Other parts of McCarthy discuss the effect of calcium administration on blood pressure, not on body weight.

As set forth in the attached Declaration, McCarthy is not prior art and the relevant passages are based on the inventors' own work. Applicants respectfully request that this rejection be withdrawn. See MPEP §§ 715.01(c) and 716.10.

**Claim rejections – 35 U.S.C. § 103(a)**

Rejection under 35 U.S.C. § 103(a) over Larsson

In the Office Action at page 6, claims 21, 23, 28, 38-40, 50 and 56-68 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Larsson, SE et al., “Low Doses of 1,25-Dihydroxycholecalciferol Increase Mature Bone Mass in Adult Normal Rats,” Clinical Orthopaedics and Related Research 127:228-235 (1977) (hereinafter “Larsson”). Applicants respectfully traverse.

Claim 38 recites a method of regulating body weight comprising administering to an individual regulating body weight an antagonist of calcitrophic hormone (1,25-(OH)<sub>2</sub>-D) activity selected from the group consisting of 1- $\beta$ , 25 dihydroxyvitamin D, a homolog of 1- $\beta$ , 25 dihydroxyvitamin D, an isomer of 1- $\beta$ , 25 dihydroxyvitamin D, and calcium, in an amount effective to block calcitrophic hormone (1,25-(OH)<sub>2</sub>-D) activity in adipocytes of said individual, said antagonist inducing weight loss, attenuating, controlling and/or reducing weight gain and/or increasing metabolic consumption of adipose tissue.

The study reported in Larsson was undertaken to “examin[e] by morphometric and quantitative methods the influence of 25-HCC [i.e., 25-hydroxycholecalciferol] and 1,25-DHCC [i.e., 1,25-dihydroxycholecalciferol] on bone mass and composition in 1-year-old rats.” (Larsson, pp. 228-29.) The 1,25-DHCC and 25-HCC administered in the Larsson study are the two active forms of vitamin D (Larsson, p. 228).

Larsson does not disclose or suggest the claimed methods because Larsson administered vitamin D (i.e., calcitrophic hormone) to the rats, whereas the claims recite an antagonist of calcitrophic hormone. The active form of vitamin D is 1 $\alpha$ , 25-dihydroxyvitamin D. See, e.g., Schwartz GG et al., “1 $\alpha$ ,25-Dihydroxyvitamin D (Calcitriol) Inhibits the Invasiveness of Human Cancer Cells,” Cancer Epidemi. Biomarkers & Prevention 6:727-732 (1997) (attached hereto as Appendix A), Abstract. It is often referred to simply as 1,25 D or by some other designation that omits the “ $\alpha$ ” symbol (see Schwartz; see also specification at paragraph 7 (referred to without indicating the alpha form) and at paragraph 24 (“alpha” designation is used when contrasting its activity with that of “beta” form). In contrast, 1 $\beta$ , 25-dihydroxyvitamin D is an antagonist of vitamin D activity. See, e.g., Norman AW et al., “Demonstration That 1 $\beta$ , 25-dihydroxyvitamin D3 Is an Antagonist of the Nongenomic but not Genomic Biological Responses and Biological Profile of the

Three A-ring Diastereomers of 1 $\alpha$ , 25-Dihydroxyvitamin D3," J. Bio. Chem. 268:20022-20030 (1993) (cited in PTO-892 attached to the pending Office Action), Abstract.

Accordingly, Larsson does not disclose or suggest the claimed methods because it does not disclose or suggest, for example, inducing weight loss, attenuating, controlling and/or reducing weight gain and/or increasing metabolic consumption of adipose tissue by administering to an individual an antagonist of vitamin D. In fact, Larsson teaches away from the claimed methods. By suggesting that administration of vitamin D may lead to weight loss, Larsson implies that an antagonist of vitamin D would not have such an effect, and further that vitamin D might lead to weight gain.

In view of the above, Applicants respectfully submit that claim 38 is patentable over Larsson. Claims 21, 23, 39-40, 50, 56-58, 61 and 63 all depend from claim 38 and are therefore allowable as depending from an allowable claim. (The other claims listed in the Office Action as rejected based on Larsson are currently either withdrawn or cancelled.) Accordingly, Applicants respectfully request that this rejection be withdrawn.

Rejection under 35 U.S.C. § 103(a) Over Larsson in view of Jequier

In the Office Action at page 8, claims 35-37 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Larsson in view of Jequier E, "Energy, Obesity, and body weight standards," Am. J. Clin. Nutr. 45:1035-47 (1987) (hereinafter "Jequier"). Applicants respectfully traverse.

Claims 35-37 depend from claim 38 and recite that the individual has Grade I, Grade II, and Grade III obesity, respectively.

As discussed above, claim 38 is patentable over Larsson because Larsson discloses weight loss after administration of vitamin D, not an antagonist to vitamin D. Because claims 35-37 depend from claim 38, they are patentable over Larsson as depending from an allowable claim.

Jequier does not cure the defects in Larsson. Jequier presents recommendations made by a panel of clinicians related to the treatment of obesity. (p. 1036). It discloses that Grade I obesity corresponds to a body mass index (BMI) of 25-29.9, Grade II obesity to a BMI of 30-40, and Grade III obesity to a BMI of greater than 40. (p. 1035). However, Jequier does not discuss vitamin D, nor does it discuss antagonists of vitamin D. Therefore, it does not disclose or suggest, for example, inducing weight loss, attenuating, controlling and/or reducing weight gain and/or increasing

metabolic consumption of adipose tissue by administering to an individual an antagonist of vitamin D. Accordingly, Jequier does not cure the above-described defects in Larsson.

Accordingly, Larsson and Jequier do not disclose or suggest the claimed methods, whether considered alone or in combination. Therefore, Applicants respectfully submit that claims 35-37 are patentable over Larsson and Jequier, and request that this rejection be withdrawn.

Rejection under 35 U.S.C. § 103(a) over McCarthy in view of Norman

In the Office Action at page 9, claims 24, 40-41, 50, 55-56, 58 and 63 are rejected under 35 U.S.C. § 103(a) as being unpatentable over McCarthy in view of Norman AW et al., “Demonstration That 1 $\beta$ , 25-dihydroxyvitamin D3 Is an Antagonist of the Nongenomic but not Genomic Biological Responses and Biological Profile of the Three A-ring Diastereomers of 1 $\alpha$ , 25-Dihydroxyvitamin D3,” J. Bio. Chem. 268:20022-20030 (1993) (hereinafter “Norman”). Applicants respectfully traverse.

As noted above, McCarthy is not prior art because it is derived from the inventor’s own work. Accordingly, claims 24, 40-41, 50, 55-56, 58 and 63 are patentable over McCarthy.

Norman does not supply the subject matter for which McCarthy was cited. Norman discusses the biological profiles of three A-ring diastereomers of 1 $\alpha$ , 25-dihydroxyvitamin D as well as 1 $\beta$ , 25-dihydroxyvitamin D (p. 20023). Each of the four tested compounds exhibited a decreased ability to stimulate transcalcachia, or intestinal transport of calcium, relative to 1 $\alpha$ , 25-dihydroxyvitamin D (pp. 20024-25). 1 $\beta$ , 25-dihydroxyvitamin D exhibited an antagonistic effect to 1 $\alpha$ , 25-dihydroxyvitamin D in relation to stimulation of transcalcachia (p. 20025, Fig. 4.). However, Norman does not disclose or suggest, for example, inducing weight loss, attenuating, controlling and/or reducing weight gain and/or increasing metabolic consumption of adipose tissue by administering to an individual an antagonist of vitamin D. Accordingly, Norman does not supply the subject matter for which McCarthy was cited.

In view of the above, Applicants respectfully submit that claims 24, 40-41, 50, 55-56, 58 and 63 are patentable over the cited references, and respectfully request that this rejection be withdrawn.

Rejection under 35 U.S.C. § 103(a) over McCarthy in view of Jequier.

In the Office Action at page 11, claims 35-37 are rejected under 35 U.S.C. § 103(a) as being

unpatentable over McCarthy in view of Jequier. Applicants respectfully traverse.

McCarthy is not prior art for the reasons set forth above in the discussion of the rejection under 35 U.S.C. § 102(a). Jequier fails to supply the subject matter for which McCarthy was cited, for the reasons set forth above in the discussion of the rejection under 35 U.S.C. § 103(a) over Larsson in view of Jequier. Accordingly, Applicants respectfully submit that claims 35-37 are patentable over McCarthy and Jequier, whether considered alone or in combination, and respectfully request that this rejection be withdrawn.

**Conclusion**

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. Accordingly, Applicants request that the Examiner issue a Notice of Allowance indicating the allowability of claims 21, 23-24, 35-38, 41, 50, 55-58, 61, 63 and 78-79 and that the application be passed to issue. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is hereby invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any additional fees which may be required for this Amendment, or credit any overpayment to deposit account no. 22-0261.

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Respectfully submitted,

Date: 11/10/08

  
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# Appendix A

## 1 $\alpha$ ,25-Dihydroxyvitamin D (Calcitriol) Inhibits the Invasiveness of Human Prostate Cancer Cells<sup>1</sup>

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### Abstract

1 $\alpha$ ,25-Dihydroxyvitamin D (1,25 D; also known as calcitriol), the hormonal form of vitamin D, can inhibit the proliferation and promote the differentiation of human prostate adenocarcinoma cells. However, little is known about the effects of 1,25 D on the invasive ability of prostate cancer cells. We used an *in vitro* bioassay of cell invasion (Amgen assay) to examine the effects of 1,25 D and a "noncalcemic" vitamin D analogue, 1,25-dihydroxy-16-ene-23-yne-cholecalciferol (16-23-D<sub>3</sub>), on the invasiveness of three well-characterized human prostate carcinoma cell lines: DU 145, PC-3, and LNCaP. PC-3 and LNCaP cells were poorly invasive in Amgen and were hardly affected by treatment with 1,25 D or 16-23-D<sub>3</sub> (<3%). Conversely, DU 145 cells were highly invasive in Amgen, and their invasion was markedly inhibited by 1,25 D and 16-23-D<sub>3</sub> (maximally 66 and 59.4%, respectively). This effect was both dose-dependent (doses of  $1 \times 10^{-7}$ – $1 \times 10^{-13}$  M) and time-dependent, with maximal inhibition at  $1 \times 10^{-7}$  M and 72 h. Significant inhibition of invasion was observed at physiological levels of 1,25 D. Neither proliferative indices nor cell cycle kinetics were altered during the experimental exposures. Treatment with 1,25 D and 16-23-D<sub>3</sub> caused a selective decrease in the secreted levels of type IV collagenases (MMP-2 and MMP-9). These findings support the hypothesis that 1,25 D reduces the risk of invasive prostate cancer and suggest a role for vitamin D compounds in the chemoprevention of invasive prostate cancer.

### Introduction

In 1996, approximately 317,000 men were diagnosed with prostate cancer, and 41,100 men died of their disease (1). Other than increasing age and black race, few consistent risk factors for prostate cancer have emerged from epidemiological studies

(2). A unique feature of adenocarcinoma of the prostate is the high prevalence of "incidental" or "autopsy" cases. Autopsy data indicate that approximately 30% of men over the age of 50 have histological evidence of prostate carcinoma and that the prevalence of these lesions reaches 60% in men over the age of 80 (3). These incidental tumors are histologically indistinguishable from those prostate cancers that ultimately threaten the lives of their hosts and are generally believed to represent an earlier stage in their natural history. Because incidental tumors are common among older men, a better understanding of factors that influence the development of clinical prostate cancer from its subclinical precursors is crucial to rational efforts at reducing prostate cancer mortality.

Considerable attention has focused on the role of vitamin D in this regard (4). There is now considerable data to support the hypothesis that the hormonal form of vitamin D maintains the differentiated phenotype of prostatic cells and that vitamin D insufficiency increases the risk for clinical prostate cancer (5, 6). United States mortality rates from prostate cancer are inversely related to the availability of UV light, the major source of vitamin D (7). This suggests that the progression of subclinical cancers to clinical cancer may be retarded by vitamin D.

Vitamin D is formed in the skin when sunlight activates its precursor, 7-dehydrocholesterol. Vitamin D then undergoes sequential hydroxylations in the liver and kidney to form 1,25 D<sub>3</sub>, the hormonal form of the vitamin. Although 1,25 D is best known for its role in calcium homeostasis, specific receptors for 1,25 D (VDRs) are present in numerous normal and malignant cells that are not involved in mineral metabolism, and 1,25 D has been shown to regulate the growth of many of these cells (8). Most human prostate cancer cell lines examined contain biologically active VDRs (9), and 1,25 D and vitamin D analogues have been shown to inhibit the proliferation and induce the differentiation of prostate cancer cells *in vitro* (10–12), in primary culture (13), and *in vivo* (14). Recent evidence indicates that nuclear VDR expression is sufficient to mediate the antiproliferative effects of 1,25 D, which occur via a genomic signaling pathway (15, 16).

Although 1,25 D is known to inhibit the proliferation of prostate cancer cells, little is known about the effect of 1,25 D on prostate cancer invasion and metastasis. Because the ability of cancerous cells to invade a basement membrane is a critical step in the evolution of tumor dissemination via hematogenous and lymphatic routes (17), we sought to investigate the effects of 1,25 D on the invasiveness of human prostate cancer cells through an artificial basement membrane-like complex ECM (Amgen). Furthermore, we sought to perform the assays in such a way that cell proliferation would not be a confounding variable.

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<sup>3</sup>The abbreviations used are: 1,25 D, 1 $\alpha$ ,25-dihydroxyvitamin D; ECM, extracellular matrix; 16-23-D<sub>3</sub>, 1,25-dihydroxy-16-ene-23-yne-cholecalciferol; TGF, transforming growth factor.

## Materials and Methods

**Cell Culture.** Human prostate adenocarcinoma DU 145, PC-3, and LNCaP cell lines were obtained from American Type Culture Collection (Rockville, MD) and were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 10,000 units/ml penicillin G, 100 µg of streptomycin sulfate (Life Technologies, Inc., Grand Island, NY), and 200 mM L-glutamine (Life Technologies). All cell lines were tested and found to be free of *Mycoplasma* contamination. Stock solutions of 1,25 D and of the vitamin D analogue 16-23-D<sub>3</sub> (Ro23-7553) were resuspended in 1% absolute ethanol. 16-23-D<sub>3</sub> was provided by Dr. Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ).

**Cell Growth and Proliferation Studies.** Tumor cells ( $1 \times 10^5$  cells/well) from each cell line were seeded onto 6-well plates, allowed to attach for 3 h, and treated with  $1 \times 10^{-7}$  M 1,25 D, an identical concentration of 16-23-D<sub>3</sub>, or 1% ethanol vehicle as control, in 2 ml of medium. After 0, 1, 2, and 3 days of incubation, cells were trypsinized, harvested, washed in PBS, and counted in a Coulter counter. In parallel, cells were labeled with [<sup>3</sup>H]thymidine (1 µCi/ml) in complete medium for the indicated time periods, and the radioactivity was measured by liquid scintillation counting. Cell viability at each end point was monitored by trypan blue exclusion. At the end of each time course, the cells were harvested from each well and counted with a Coulter counter.

**Flow Cytometry Analysis.** Cells from the control and treated groups were subjected to cytofluorometric analysis using a Cycle TEST Plus DNA reagent kit (Becton Dickinson). Briefly, cells ( $2 \times 10^6$  cells/ml) were washed, harvested in citrate buffer, and incubated with a trypsin-RNase mixture, followed by staining with 0.5% propidium iodide. Cell cycle parameters were obtained by a FACScan flow cytometer and analyzed using Mod FIT software. Approximately 20,000 ungated events were collected and presented as histograms.

**In Vitro Human Invasion (Amgel) Bioassay System.** The characteristics of Amgel have been described previously. Briefly, Amgel is a processed yet physiological human ECM derived from a nontumor tissue source, acellular amniotic (18). The major components of Amgel include collagens I and IV, laminin, entactin, tenascin, and heparan sulfate proteoglycan. Neither the growth factors epidermal growth factor, platelet-derived growth factor, TGFβ1, TGFβ2, and fibroblast growth factor nor the  $M_r$  72,000/ $M_r$  92,000 metalloproteases (type IV collagenases/gelatinases) are detectable in this biomatrix. Amgel maintains a stable gel-like matrix architecture both at 4 and 37°C. Amgel-based coculture systems that mimic a "defined" human cell-matrix environment and allow dissection of tumor cell adhesion, migration, and matrix-degradation steps of the invasion cascade (19–21).

The invasion assay protocol used Amgel-coated inert filters (8-µm pore size; Poretics Corp., Livermore, CA) as an extracellular membrane barrier sandwiched between custom-made 6-mm diameter Lucite chambers. Details of this 72-h assay, including the coating of Amgel onto filters and the incubation and recovery of [<sup>3</sup>H]thymidine-labeled cells, have been described by Siegal *et al.* (18). Briefly, stock Amgel (4 mg of protein/ml) was prepared in our laboratory, diluted with 1 × PBS and 1 M HEPES (1:10.5, v/v), and coated onto the filters. The Amgel-coated filters were then rehydrated for 1 h in serum-free medium using constant agitation. Cells growing in logarithmic phase were labeled with 1 µCi/ml [<sup>3</sup>H]thymidine (60 Ci/mmol) for 24 h and washed thoroughly to remove

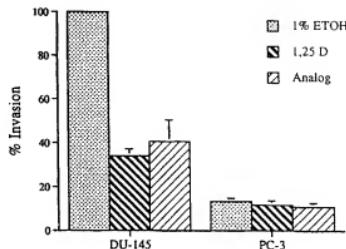


Fig. 1. Invasiveness of DU 145 and PC-3 cells treated with  $1 \times 10^{-7}$  M 1,25 D or 16-23-D<sub>3</sub>. Control invasion of DU 145 cells was set at 100%. Columns, means of three experiments performed in quadruplicate; bars, SE.

unincorporated radioactivity, and their specific gravity was determined.

Labeled cells ( $7 \times 10^6$ ) that were resuspended in 0.4 ml of RPMI with 0.1% BSA were seeded onto rehydrated Amgel filters (70 µg of protein), and the lower chambers were filled with 2.5 ml of medium containing 5% dialyzed fetal bovine serum. Cells were incubated at 37°C for 6 h to foster cell attachment. The following day, the medium in the chambers was replaced with medium containing various concentrations of 1,25 D or 16-23-D<sub>3</sub> ( $1 \times 10^{-7}$ – $1 \times 10^{-13}$  M). The medium in the top chambers was replaced at 24- and 48-h time intervals with fresh medium containing the test compounds, and incubation was continued for a total of 72 h. The cells traversing through the Amgel-coated filters were collected by treatment of lower chambers and the underside of the coated filters with 0.25% trypsin, followed by 1× PBS washes after trypsinizing. The cells from the lower chamber (*i.e.*, cells treated with initial medium, a trypsin wash, and PBS washes) were collected by vacuum filtration onto a 0.45-µm Millipore filter to resolve cell-bound and free radioactivity. The radioactivity of filter-bound cells was measured by liquid ( $\beta$ ) scintillation counting. Percentage of invasion was determined by formula as follows: postcounting/precounting × 100. This method of quantitation provided highly reproducible results and proved advantageous over other techniques of cell counting.

**Gelatin Zymography.** Tumor cells were grown to confluence, washed twice with 1 × PBS, and then cultured in serum-free medium for 48 h in the presence or absence of  $1 \times 10^{-7}$  M 1,25 D or 16-23-D<sub>3</sub>. Conditioned media were concentrated by a 0–60% ammonium sulfate precipitation, dialyzed, and dissolved in SDS-PAGE buffer without a reducing agent. Samples (40 µg of protein), without heating, were resolved on 10% separation/4% stacking acrylamide gels copolymerized with gelatin (0.1%). HT-1080 human fibrosarcoma cells were used as a positive control. Gels were run at a constant current and voltage (40 mA and 150 V) for 3 h at 4°C. SDS was removed by rinsing the gels in 2.5% Triton X-100, followed by an overnight incubation in 50 mM CaCl<sub>2</sub>, 2 µM ZnCl<sub>2</sub>, and 1% Triton X-100 at room temperature. Finally, gels were stained with 0.1% amido black, destained, and analyzed by densitometry. The molecular weight markers were always reduced and included on the same gels for the estimation of size/position.

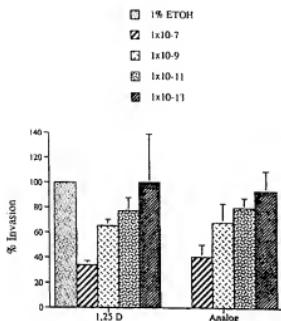


Fig. 2. Invasiveness of DU 145 cells that were exposed to different concentrations of 1,25 D and 16-23-D<sub>3</sub>. As the concentration of the two compounds decreased, the invasion rates increased, reaching the control level at  $1 \times 10^{-13} M$ . Columns, means of five experiments performed in quadruplicate; bars, SE.

## Results

Three well-characterized human prostate adenocarcinoma cell lines were exposed to  $1 \times 10^{-7} M$  1,25 D or  $1 \times 10^{-7} M$  16-23-D<sub>3</sub>, and their invasive ability was determined by use of a 72-h *in vitro* invasion assay. As seen in Fig. 1, 1,25 D and 16-23-D<sub>3</sub> decreased the invasion rate of DU 145 cells (mean  $\pm$  SE) to  $34.0 \pm 3.20\%$  and  $40.6 \pm 9.84\%$ , respectively. In comparison with DU 145 cells, PC-3 cells had an invasion rate through Amgel of only  $13.3 \pm 1.57\%$ . In the presence of an equal concentration of 1,25 D and 16-23-D<sub>3</sub>, this invasive ability was reduced minimally, to  $11.6 \pm 2.04\%$  and  $10.8 \pm 1.76\%$ , respectively. Additionally, no significant changes were noted in the presence of the test compounds with LNCaP tumor cells (data not shown).

Because 1,25 D and its synthetic analogue selectively inhibited the invasive ability of human prostate cancer cells, we next sought to determine whether this response was dose dependent. In Fig. 2, we demonstrate that, indeed, this response was sensitive to dosage manipulation with either compound, with the response being completely ablated at  $1 \times 10^{-13} M$ . (For reference,  $10^{-7} M$  1,25 D is a pharmacological dose;  $10^{-10} M$  is a physiological dose; and  $10^{-13} M$  is a subphysiological dose.) The anti-invasive effect was also time dependent, with maximal inhibition at 72 h (data not shown).

Because previous studies have demonstrated an antiproliferative effect of 1,25 D and various analogues, growth curves and flow cytometric analysis were performed to determine whether the anti-invasive effects we observed could be accounted for either in altered proliferative rates or modulation of the cell cycle. As seen in Fig. 3, DU 145 cell counts as determined over 72 h show nearly identical growth kinetics in the presence or absence of drug. The results of automated cell counting were superimposable on the results obtained by scintillation counting of cell-bound radioactivity. Similarly, PC-3 and LNCaP exhibited nearly identical results (data not shown).

When cells were incubated for greater than 24 h in either 1,25 D or 16-23-D<sub>3</sub> and subjected to fluorescence-activated cell sorting analysis, the three histograms were again nearly super-

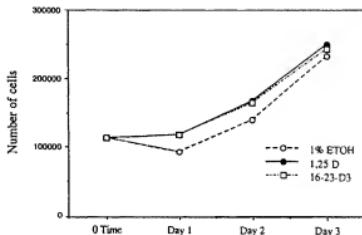


Fig. 3. Growth curve of DU 145 cells in the presence of  $1 \times 10^{-7} M$  1,25 D,  $1 \times 10^{-7} M$  16-23-D<sub>3</sub>, or ethanol vehicle. Compared to the control, even at this high concentration, neither 1,25 D nor 16-23-D<sub>3</sub> had any effect on the growth rate of DU 145 cells over the 3-day time course. Data points, means of two experiments performed in duplicate.

imposable. The DU 145 cells were determined to be diploid with a  $G_0/G_1$  corresponding to  $56.3 \pm 1.6\%$ , a  $G_2/M$  of  $17.1\% \pm 0.5\%$ , and a total percent S-phase fraction (representing the actively dividing cells) of  $26.7 \pm 1.9\%$  (Fig. 4). The coefficient of variation was very narrow, at  $3.58 \pm 0.07\%$ . Therefore, we conclude that 1,25 D and 16-23-D<sub>3</sub> did not alter the growth rates or the cell cycle parameters in DU-145, PC-3, and LNCaP cell lines under short-term (48 h) experimental conditions. Thus, neither cell cycle nor growth rate effects can account for the dramatic results obtained in the Amgel invasion assays.

Last, in an attempt to understand the mechanism whereby 1,25 D and its congener modulate the invasive phenotype of DU 145 cells, we examined the secreted type IV collagenase levels by zymography in the presence or absence of the two compounds. In Fig. 5, the gelatinolytic activity of MMP-2 and MMP-9 were seen to be markedly decreased in the presence of both drugs, supporting the hypothesis that calcitriol and its synthetic analogue inhibit MMP activity, which, in turn, reduces proteolytic digestion of the ECM, resulting in decreased invasive ability.

## Discussion

The principal clinical problem in prostate cancer is metastatic disease (22). Because invasion of the basement membrane is a critical step in the metastatic cascade, agents that modify the invasive phenotype have obvious potential as anticancer drugs. The present study demonstrates that 1,25 D and the synthetic analogue 16-23-D<sub>3</sub> significantly inhibit the invasive phenotype of human prostate cancer cells. This inhibition of invasion was independent of any effect on cell proliferation and was associated with a decrease in the production of proteolytic enzymes.

The ability of a cell to metastasize *in vivo* is highly complex and multifactorial. It has been shown in multiple systems, however, that, compared to poorly metastatic clones, highly metastatic cell populations often demonstrate increased invasive ability. Invasive cell behavior has been represented as a three-step model. Step 1 involves tumor cell attachment to basement membrane via cell surface receptors, such as the laminin receptor. Step 2 involves the proteolytic degradation of the matrix by metalloproteinases or other enzymes, and step 3 involves the active migration of cells through the disintegrated ECM to distant sites (23, 24).

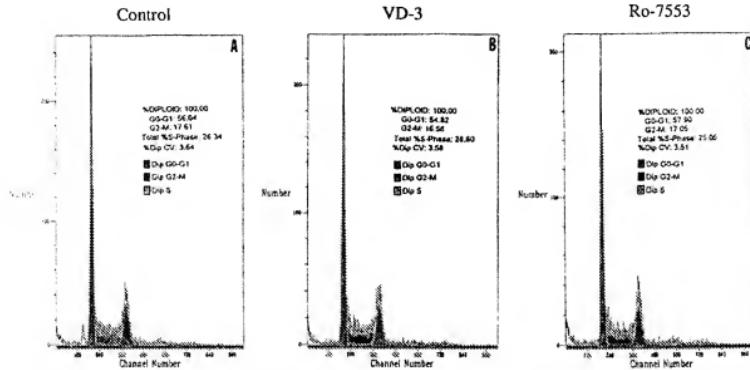


Fig. 4. Effect of 1,25 D and 16-23-D<sub>3</sub> on cell cycle kinetics of human prostate DU 145 adenocarcinoma cells. Cells were incubated with or without  $1 \times 10^{-7}$  M 1,25 D or  $1 \times 10^{-7}$  M 16-23-D<sub>3</sub> for 48 h and subjected to fluorescence-activated cell sorting analysis. G<sub>0</sub>-G<sub>1</sub> (2C DNA) and G<sub>2</sub>-M (4C DNA) cell cycles are shown, as is the S-phase population representing the actively replicating cells. No difference is apparent among the three groups.

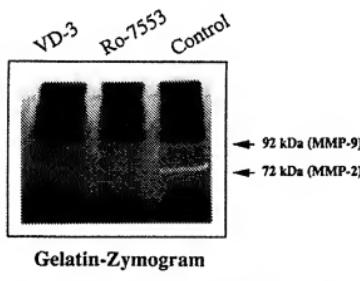


Fig. 5. Effect of 1,25 D and 16-23-D<sub>3</sub> on type IV collagenases (MMP-2/MMP-9) expression and secretion. DU 145 tumor cells were cultured in serum-free medium for 48 h with or without  $1 \times 10^{-7}$  M 1,25 D or  $1 \times 10^{-7}$  M 16-23-D<sub>3</sub>. Concentrated medium was analyzed by gelatin-embedded SDS-PAGE zymography. Clear bands, gelatinolytic activity of MMP-2 ( $M_r$ , 72,000 collagenase) and MMP-9 ( $M_r$ , 92,000 collagenase).

Matrix metalloproteinases, especially  $M_r$  72,000 (MMP-2) and  $M_r$  92,000 (MMP-9) type IV collagenases, are candidate proteases that are thought to play a central role in tumor invasion and metastasis by acting at step 2 of invasion (see above; Ref. 25). MMPs are initially produced as proenzymes, and only after activation are they secreted into the extracellular milieu. MMP-2 is known to localize to invasive cell membrane surfaces secondary to its direct binding to members of the integrin cell adhesion protein family (26). We used 0.1% gelatin-based zymography to measure the levels of  $M_r$  72,000 and

$M_r$  92,000 gelatinases in serum-free conditioned medium, having previously demonstrated a close relationship between MMP-2 activity and the invasive index of tumor cells as seen in the Amgel assay. Recently, Stearns and Stearns (27, 28) reported that the activated type IV collagenase, MMP-2a, was expressed in prostate cancer and that increased expression was associated with neoplastic progression. In a series of studies using SDS-PAGE, Western blotting, zymography, quantitative ELISAs, and quantitative computer-assisted imaging analysis of immunohistochemically stained sections, increasing levels of MMP-2a were identified as the Gleason grade of the prostate cancer increased, indicating an inverse relationship between differentiated state and enzyme level.

A number of studies have used intact human basement amion or Matrigel-based reconstituted basement membrane in *in vitro* invasion assays systems to study invasive ability of prostate and other transformed human cells (29, 30). Hoosine *et al.* (31) reported that the presence of cell surface urokinase receptors on PC-3 and DU 145 cells correlated with high invasive ability through short-term exposure to Matrigel, whereas LNCaP cells, which lack urokinase receptors, had poor invasive ability. However, Matrigel has several limitations with regard to its use in human systems. First, Matrigel is prepared from malignant mouse (EHS) tumor and contains numerous growth and differentiation factors as well as proteases. The presence of these factors makes the interpretation of observed cell-matrix interactions more complex and the subject of controversy (32-34). Second, Matrigel contains little or no types I and IV collagen, the essential footprint for epithelial cell-ECM interactions. Unlike Matrigel, Amgel represents a predominantly growth factor- and collagenase-free human ECM environment. Thus, we believe that the use of Amgel represents a methodological advance over Matrigel-based assay systems for the study of human cancer cell invasion.

In addition to 1,25 D, other steroids (e.g., estradiol-17 $\beta$

and progesterone) and growth factors (epidermal growth factor, TGF $\alpha$ , and TGF $\beta$ ) have been demonstrated to influence migration and invasion of human transformed cells, at least in part, by up- or down-regulating type IV collagenases (35). Interestingly, it has been reported that extracellular organelles produced by cells during matrix mineralization contain metalloproteases that are regulated by vitamin D metabolites (36). This suggests that vitamin D may play a fundamental role in cell-matrix interactions.

Our observations in human prostate cancer cells are similar to those of Hansen *et al.* (37), who demonstrated a dose-dependent inhibition of both migration and invasive ability of MDA-MB-231 human breast cancer cells *in vitro* in the presence of 1,25 D over a 4-day period in which no antiproliferative effect was noted (37). Similarly, Young *et al.* (38) have shown that 1,25 D reduces tumor metastasis and recurrence in mice carrying Lewis lung carcinoma (38). Further, they presented evidence that this was accomplished through modulation of the myelopoiesis-associated immunosuppressive cascade (39). This is not to say that 1,25 D is not antiproliferative. In fact, at least six recent publications have presented evidence of such an effect (11, 13, 15, 16). Rather, it was the specialized circumstances of experimental conditions linked to short-term exposures that allowed the separation of anti-invasive from antiproliferative effects to be examined in this study.

A logical next step from this *in vitro* study would be to use vitamin D analogues in an *in vivo* model of metastatic prostate cancer. Such a study is presently underway in the authors' laboratory (40). However, this work is tempered by the knowledge that the clinical use of oral 1,25 D (1.5  $\mu$ g p.o.) in the treatment of metastatic prostate cancer has been explored in a Phase II trial (41). No objective responses were observed (defined as a 50% sustained decrease in serum prostate-specific antigen). However, two men experienced transient decreases in prostate-specific antigen of 25 and 45%. The major toxicity was hypercalcemia. These findings suggest that, in at least some men, 1,25 D is bioactive in advanced prostate cancer and that a "noncalemic" analogue of 1,25 D may be an attractive candidate for future clinical trials.

In summary, we report that 1,25 D and a vitamin D analogue significantly inhibit the invasion of DU 145 human prostate cancer cells in a dose-dependent fashion. Consistent findings demonstrating inhibition of prostate cancer cell invasion in Matrigel by 1,25 D has recently been reported (in an abstract) by other investigators.<sup>4</sup> The anti-invasion effect we observed is apparent at physiological levels of 1,25 D (*i.e.*, 10<sup>-10</sup> M) and is correlated with a decreased ability of prostate cancer cells to manufacture matrix-degrading proteases. We conclude that the antitumor actions of 1,25 D in human prostate cancer involves, in part, the inhibition of cell invasiveness. These findings support the hypothesis that 1,25 D reduces the risk of invasive prostate cancer and suggest a potential role for vitamin D compounds in the chemoprevention of invasive prostate cancer (42).

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